Vascular Injury: Quantification of Experimental Focal Endothelial Denudation in Rats Using Indium-111-Labeled Platelets

Michael A. Reidy, Keizo Yoshida, Laurence A. Harker, and Stephen M. Schwartz

Rat platelets were labeled with indium-111 (\(^{111}\text{In}\)) and injected into rats whose aortas were subjected to endothelial denudation. A plot of \(^{111}\text{In}\) activity vs the number of missing endothelial cells showed a direct correlation between these parameters and it was possible to detect endothelial denudation when approximately 600 cells were removed. These studies also revealed that intact adjacent endothelium from aortas subjected to injury had detectably increased \(^{111}\text{In}\) activity compared with endothelium of control, uninjured rats. This activity was not found if \(^{111}\text{In}\)-platelets were preincubated with acetylsalicylic acid (1.3 mM) prior to injection and subsequent denudation procedure. To test if \(^{111}\text{In}\) platelet activity could be transported to sites distant from the site of injury, a small catheter (PE 50) was passed down the carotid artery so that it reached the aortic arch or inserted via a femoral artery to the iliac-aortic bifurcation. Significant increases in \(^{111}\text{In}\) activity were detected in the lower thoracic aorta when the catheter was introduced into the aortic arch but not when the catheter was introduced via the femoral artery. Morphologic evaluation of these sites showed no endothelial cell loss or adhering platelets. These studies suggest that detectable uptake of released platelet products may occur at distal sites of intact endothelium.


Vascular injury causing endothelial cell loss is generally documented by various morphological techniques.\(^1\)\(^-\)\(^4\) However, when the extent of injury is minimal and its location is unknown, even an extensive morphological search may fail to demonstrate endothelial cell loss. Two alternative approaches to detect endothelial cell loss involve the indirect measurement of accelerated removal of radiolabeled platelets from blood or the direct determination of isotopically labeled platelet deposition in a particular vascular bed or organ.\(^5\)\(^-\)\(^9\) Some data are available regarding platelet consumption with respect to the amount of denuded surfaces,\(^8\)\(^,\)\(^9\) but the relationship between the deposition of radiolabeled platelets on injured vessels and the luminal area showing endothelial denudation has not been previously examined.

In this study we have used \(^{111}\text{In}\)-labeled platelets to determine the correlation between endothelial cell loss and platelet deposition on the denuded vessel wall. The study shows a linear relationship between the number of experimentally denuded endothelial cells and the quantity of \(^{111}\text{In}\)-platelet activity deposited in the vessel wall. Using this approach, we could detect denudation involving approximately 0.4% of the endothelial cell surface in a vascular segment. However, the data also show that detectable platelets or platelet products are associated with intact endothelium following vascular injury at proximal sites; this uptake of \(^{111}\text{In}\)-platelet activity by endothelium is prevented by aspirin treatment of the labeled platelets before infusion.

Methods

Experimental Design

Seventy male Sprague-Dawley rats 350 to 450 g in body weight were used in these experiments. Groups of 10 animals each were used for a given size of injury, and 10 unmanipulated animals were used as controls. An additional 10 animals were used in the noninjuring catheter study, and the remaining animals were used in the aspirin study.

\(^{111}\text{In}\)-labeled platelets were injected into rats via the tail vein 1 hour before surgery. Immediately after endothelial

Michael A. Reidy and Stephen M. Schwartz are at the Department of Pathology, University of Washington, Seattle, Washington. Keizo Yoshida is at the Fujisawa Pharmaceutical Company, Limited, Fermentation Research Laboratories, Osaka, Japan. Laurence A. Harker is at the Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California.

This work was supported in part by National Institutes of Health Grants HL-31950 and HL-30203 and American Heart Association Grant 81-837.

Address for reprints: Michael A. Reidy, Department of Pathology, SJ-60, University of Washington, Seattle, Washington 98195.

Michael A. Gimbrone, Jr. kindly acted as Guest Editor for this manuscript.

Received June 21, 1985; revision accepted December 28, 1985.
injury, each animal was injected with Evans blue dye (0.5 ml of 1% solution). Ten minutes after aortic denudation, each animal was killed and perfusion fixed with 2% glutaraldehyde and 1% paraformaldehyde in phosphate buffer as described previously.10 The aorta was removed and cleared of periadventitial fat; a 2 cm length of the lower thoracic aorta was taken for gamma counting. Subsequently, the aortas were opened to expose the luminal surface and pinned onto teflon sheets. The tissue was dehydrated through a series of alcohol and dried in a critical-point dryer. The extent of endothelial cell loss in each segment was quantitated using the scanning electron microscope. This was accomplished by determining the number of fields with no endothelial cells and quantitating the average number of endothelial cells found in a field of identical magnification.

**Endothelial Injury**

Endothelial cells were removed from the thoracic aortas by using either the microscraping technique10 or a balloon catheter. The smallest injury was made with a 3/0 nylon microfilament inside a length of PE 50 tubing that was passed into the thoracic aorta through the femoral artery. The end of the filament was angled to 45° so that, by rotating in a circular fashion, a circumferential zone of endothelium was removed. Alternatively, by pulling the filament lengthwise along the vessel, a linear zone of endothelium three to five cells wide was removed. A larger injury was made by substituting a thicker filament. Total denudation of aorta was achieved using an inflated 2F embolectomy catheter.4

In an additional four animals, acetylsalicylic acid (ASA)-treated 111In-platelets were reinjected, and 1 hour later a zone of endothelial denudation was made using the larger filament. The animals were then injected with 0.4 ml of Evans blue dye (1% in saline), and 10 to 15 minutes later each animal was killed as described above. Four uninjured animals were also injected with ASA-treated platelets and served as controls. The deendothelialized regions that were stained by Evans blue were cut and counted separately from the endothelialized region of the aorta.

To determine the effects of the catheter alone in the absence of aortic injury, rats were injected with 111In-labeled platelets as above, and PE 50 tubing was inserted via the left carotid artery to the aortic arch or via the femoral artery to the aortic bifurcation. In each instance, the tubing was left in place for approximately 30 to 45 seconds and then withdrawn. Animals were then perfusion-fixed and a 2 cm segment of thoracic aorta proximal to the diaphragm was removed and counted. After gamma counting, these segments were processed for scanning electron microscopy.

**Labeling of Platelets with Indium-111**

Rat blood (9 ml) was drawn from donor rats via cardiac puncture into a syringe containing 1 ml of acid citrate dextrose (ACD) solution. After mixing gently, the blood was centrifuged for 10 minutes at 350 g and the platelet-rich plasma (PRP) was removed from the red cell pellet. The PRP was then centrifuged at 1000 g for an additional 15 minutes to form a platelet pellet; the supernatant plasma was removed and saved. The platelet pellet was washed with Ringer's citrate dextrose (RCD) and was resuspended in 1.5 ml of RCD. A sample (3 μl) of this suspension was taken for Coulter cell counting.111In-oxine solution (500 μCi, Amersham) was added to the platelet suspension and was incubated for 20 minutes at room temperature. A further sample (3 μl) was then taken for counting radioactivity. The 111In-platelet pellet was reformed by centrifuging the platelet suspension at 1000 g for 15 minutes, and the supernatant was discarded. The platelet pellet was washed with 2 ml of RCD, and the platelets were resuspended in rat plasma. A sample of this suspension (3 μl) was removed to determine labeling efficiency (see below). Each rat was injected with 0.5 ml of the 111In-labeled platelets, and 1 hour later a blood sample (100 μl) was obtained. This sample was centrifuged for 30 minutes at 3000 g, and the supernatant was separated from the platelet pellet. Both samples were then counted for indium activity.

In the ASA experiment, platelets were prepared as above and incubated with ASA (1.3 mM).11 ASA was dissolved in pure ethanol (180 mg/ml) and was added to the platelet pellet so that the final concentration was 1.3 mM. After incubation for 30 minutes, the pellet was washed with RCD and resuspended in plasma as described above. To ensure that the above concentration of ASA would inhibit platelet release, ionophore A23187 (0.1 mg/ml in dimethyl sulfoxide, DMSO) was added to those platelets that had previously been incubated with ASA.

**Preparation of Supernatant of Indium-111 Platelets**

Following the resuspension of the platelet pellet in plasma, the platelet suspension was recentrifuged for 15 minutes at 3000 g to form supernatant that was essentially platelet-free. The resultant supernatant was removed and a known volume was injected into both denuded and unmanipulated rats.

To test whether released products from the platelets could react with the injured arteries, platelets were labeled with 111In as above and then were incubated with 0.1 ml of ionophore A23187 (0.1 mg/ml in DMSO). After 20 minutes at 37° C, the samples were centrifuged at 3000 g for 30 minutes and the supernatant was removed. This supernatant was then injected directly into the aorta via a PE 10 catheter at the same time as the aorta was subjected to a denuding injury. Control animals were not injured but did receive an equal volume of platelet supernatant or received the 111In-labeled platelets alone. After 10 minutes, all animals were killed as detailed and a 2 cm length of thoracic aorta was counted for activity.

To determine the releasable activity in rat platelets, 0.2 ml of PRP was incubated with the 0.1 ml of ionophore A23187 (0.1 mg/ml in DMSO) or with collagen, 0.1 and 0.5 μg/ml (Kollagen reagent Horm, Hormon-Chemie, Munich, Germany). After 20 minutes at 37° C with gentle agitation but not stirring, the samples were centrifuged at 3000 g for 30 minutes and the supernatant was removed.
The $^{111}$In activity of both the platelet pellet and the supernatant was then counted in a gamma counter.

**Calculations**

Platelet labeling efficiency was expressed as the proportion of platelet-bound $^{111}$In injected into each rat and was calculated as follows:

\[
\text{Labeling efficiency of platelets} = \frac{\text{activity in platelet pellet}}{\text{total activity of PRP}} \tag{1}
\]

Throughout the experiments the calculated efficiency was 94% ± 3%.

The $^{111}$In activity of each aorta was calculated from a 2 cm length of both injured and uninjured vessels and was expressed as cpm/1 cm length of aorta:

\[
\text{activity in aorta} = \frac{\text{cpm of aorta} - \text{cpm background}}{\text{cpm of total $^{111}$In-platelet injected activity}} \tag{2}
\]

The activity of injured aortas was then expressed as the fold increase above the control aortas.

**Autoradiography**

Tissue to be used for autoradiography was perfusion-fixed as described, immersion-fixed for 1 hour, and then washed three times in glycine buffer (0.1 M). The vessel

![Figure 1](http://atvb.ahajournals.org/) Scanning electron micrographs of injured rat aortas. A. Small longitudinal injury in which a zone of approximately three to five endothelial cells wide was removed. x 140. B. Larger longitudinal injury in which a zone of approximately 10 to 15 endothelial cells wide was removed. x 80. C. Circular injury across aorta in which a zone of approximately two cells wide was removed. x 150. D. View of denuded surface after injury with nylon filament. A monolayer of platelets covered the exposed subendothelium. x 2400.
was cut open and pinned to Teflon sheets, was dehydrated, and then dried as described above. These preparations were dipped in autoradiographic emulsion (Ilford K5) and exposed for 1 week. After development and fixation, the specimens were again dried and viewed by scanning electron microscopy.

**Results**

**Endothelial Injury**

The results of denudation by catheters varying from small filaments to the 2F balloon catheter are shown in Figure 1. The balloon totally denuded the thoracic aorta, while the large catheter removed a longitudinal zone of cells approximately 10 to 15 cells wide. The small 3/0 nylon monofilament removed a longitudinal zone three to five cells wide or a circumferential zone one to three cells wide (Figure 1). In all cases, platelets adhered to the denuded surfaces but not to the adjacent intact endothelium. Moreover, following the injection of Evans blue, the blue area correlated well with the denuded regions.

**Indium-111 Activity of Blood**

To ensure that the indium activity in the blood was associated with the platelets and not plasma components, blood samples were drawn from each animal 1 hour after platelet injection. These samples were centrifuged, and the platelet pellet activity was calculated. In all the experiments in this study, the range of pellet activity varied between 92% to 96%. For example, in one experiment with eight rats, the mean platelet activity was 93.3% ± 1.9% (SD). To monitor if any increase in nonplatelet 111In activity was present after injury, blood samples were removed from six animals immediately before and after injury to the aorta. The time elapsed between withdrawing these two samples was 10 minutes. The activity in the platelet supernatant was 0.09% ± 0.01% (SD) before surgery and 0.11% ± 0.03% (SD) after surgery. These values were not significantly different.

**Indium-111 Activity in Injured Artery**

Because 111In decays rapidly, measurements of 111In activity in the injured aortas were normalized against control vessels. The fold increase in 111In activity produced by the various sized injuries shows a linear correlation ($r = 0.85$) with the proportion of the surface denuded endothelial cells (Figure 2). The results also show that measurements of 111In activity of the aortas detected the removal of as few as 600 endothelial cells over an area of 1.5 mm$^2$. In terms of a rat thoracic aorta, these data show that endothelial injury can be detected by this procedure for injuries involving loss of less than 1% of the endothelium. Since the actual number of missing cells varied for the circumferential wound, thereby making it difficult to group these values, a single value is shown in Figure 2 to illustrate the sensitivity of the procedure.

Of interest is the observation that the extrapolation of 111In aortic activity to no cell loss (Figure 2) does not go through unity as would be predicted in the absence of endothelial cell denudation. Since this plot presents the results of complete 2 cm-long segments of injured aorta, the aortic segments counted include regions of intact as well as denuded endothelium with exception of the balloononed vessels. When the completely denuded areas of aorta were counted separately, the plot of 111In activity against endothelial cell loss intercepted the ordinate at approximately unity (Figure 2). This suggests that an in-
crease in the number of counts occurs over the intact endothelium and that the extent of this increase is constant and independent of the extent of denudation. The $^{111}$In activity of the injured section alone was still significantly different from the control aortas ($p < 0.01$) as well as from the combined $^{111}$In activity of the injured plus uninjured sections of the same 2 cm length of aorta ($p < 0.01$). This suggests that for small injuries, as much as 50% of the total net $^{111}$In activity is apparently localized to areas of aorta with intact endothelium. This activity can be visualized by autoradiography as shown in Figure 3 in which silver grains over intact endothelium are evident. Since the Auger electrons, and not the garjima emission, expose the silver grains, one can say that a 50% increase in the number of counts occurs over the intact endothelium and that the extent of this increase is constant and independent of the extent of denudation. The $^{111}$In activity of the injured section alone was still significantly different from the control aortas ($p < 0.01$) as well as from the combined $^{111}$In activity of the injured plus uninjured sections of the same 2 cm length of aorta ($p < 0.01$). This suggests that for small injuries, as much as 50% of the total net $^{111}$In activity is apparently localized to areas of aorta with intact endothelium. This activity can be visualized by autoradiography as shown in Figure 3 in which silver grains over intact endothelium are evident. Since the Auger electrons, and not the gamma emission, expose the autoradiographic emulsion, relatively little activity can be visualized with this procedure.

In each preparation, approximately 6% of the injected $^{111}$In was not bound to platelets. Therefore, to ensure that the $^{111}$In activity in the denuded vessels was derived from platelets and was not just a consequence of increased permeability of nonplatelet-bound $^{111}$In, the injury experiment was repeated with only the nonplatelet $^{111}$In supernatant fraction injected. The results are shown in Figures 2 and 4. Regardless of the extent of injury, the increase in the $^{111}$In activity in the aorta was the same as control values.

To explain the increased activity over the intact endothelium, an experiment was conducted to assess whether without denudation, the presence of the catheter could result in uptake of $^{111}$In activity released from activated intravascular platelets at a site remote from the location of the catheter. Therefore, in a group of rats which had received $^{111}$In platelets, a length of PE 50 (which is normally used as a sheath to introduce the nylon filaments into the thoracic aorta) was passed either down the carotid to the level of the aorta or via a femoral artery to the iliac aortic bifurcation. The $^{111}$In activity in 1 cm segments of thoracic aorta is shown in Table 1. An increase in the aorta $^{111}$In activity approximately twice that of the background was observed when the catheter was introduced via the carotid artery as compared to the activity from control, unmanipulated aortas. It should be emphasized that this segment of aorta, taken for estimation of $^{111}$In activity, was at least 3 cm away from the catheter tip and by scanning electron microscopy showed no loss of endothelium or platelet adhesion. No increase in activity was seen when the catheter was introduced via the femoral artery. Since the increased activity was measured in a segment at least 3 cm away from the carotid catheter, these data again suggest some sort of transfer of label from platelets to the intact, endothelialized wall.

Table 1. $^{111}$In Activity in Lower Thoracic Aorta after Insertion of Catheter into Carotid or Femoral Artery

<table>
<thead>
<tr>
<th>Catheter placement</th>
<th>Aortic activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter inserted via carotid artery (n = 6)</td>
<td>2.2 ± 0.6†</td>
</tr>
<tr>
<td>Catheter inserted via femoral artery (n = 6)</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

The activity of indium in each aorta was determined from a 1 cm length of thoracic aorta immediately above the diaphragm. This site was distant from the catheter and showed no endothelial cell loss.

*Expressed as fold increase above background.
†$p < 0.005$.

Table 2. Indium Activity in Platelet Releasate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{111}$In activity in supernatant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (n = 6)</td>
<td>(1 µg/ml) 33 ± 2 (0.5 µg/ml) 31 ± 3</td>
</tr>
<tr>
<td>A23187 (0.1 mg/ml) in dimethyl sulfoxide (n = 6)</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (n = 6)</td>
<td>0.1 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3. Effect of Platelet Incubation with Aspirin on Indium Activity of Injured Aortas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{111}$In activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin treated</td>
<td>Endothelialized zone: injured aorta 1.2 ± 0.1</td>
</tr>
<tr>
<td>Untreated</td>
<td>Denuded zone: injured aorta 5.6 ± 2.9</td>
</tr>
</tbody>
</table>

Platelets were incubated with aspirin (1.3 mM) and injected into rats. One hour later the aortas of one group of animals were injured by using a denuding nylon catheter while the other group served as controls. Each animal then received an intravenous injection of Evans blue dye (1% in saline) and killed 10 to 15 minutes later. Zones of denudation stained blue and were separated from the remainder of the aorta. Equal areas of denuded (blue) and endothelialized (white) aortas were compared to the values of equal areas of control aortas.

*Expressed as fold increase above indium activity of control tissue.
Since rat platelets release an excess of 26% of the bound $^{111}$In activity after incubation with the ionophore A23187 or with collagen (Table 2), we examined the additional possibility that the increased aortic $^{111}$In activity was derived from platelet releasate. In this experiment, the platelet releasate was obtained by incubating $^{111}$In-labeled platelets with the ionophore A23187 and then injecting it into animals subjected to injury with the nylon catheter. Since released products from the nonlabeled platelets in vivo might block the binding of this injected labeled material in a competitive fashion, this $^{111}$In-released material was injected directly into the aorta at the same time as endothelial denudation. The $^{111}$In activity of the injured aortas was identical to that obtained from aortas from control uninjured animals.

Since in the above study the injected nonplatelet-bound $^{111}$In activity may be immediately bound by plasma proteins and thereby prevented from interacting with the vessels, we next asked whether inhibition of platelet release in vivo would prevent the localization of $^{111}$In activity over intact endothelium in injured aortas. Consequently, platelets were preincubated with ASA (1.3 mM) before injection. As stated above, addition of ionophore A23187 caused a release of 26% of the $^{111}$In activity for rat platelets, but when preincubated with ASA (1.3 mM), the released activity was equivalent to that found in platelets not incubated with A23187 (25.9% ± 5.9% vs. 12.1% ± 5.1%). Control, uninjured rats were injected with the same ASA-treated platelets. No increase in $^{111}$In activity was found over the completely endothelialized areas, and the actual $^{111}$In aortic activity in these segments was equal to that of controls (Table 3). The activity over the injured zone, however, was still increased. Thus, aspirin inhibited the uptake of counts by the intact endothelium.

**Discussion**

Detection of endothelial injury by measuring increased deposition of radiolabeled platelets with $^{51}$Cr or $^{111}$In in regions of the vasculature has been reported previously. In the present studies, a linear relationship is documented between the deposited platelet isotopic activity in the vessels and the extent of endothelial loss. By removing defined amounts of endothelium, we have shown that this procedure can detect an endothelial loss of approximately 600 cells over a surface area of 1.5 mm². Although this might appear to be somewhat insensitive compared to scanning electron microscopy, in terms of the entire endothelial cell population of the rat thoracic aorta, this means that denudation involving as little as 0.4% of the endothelial cells can be detected with this technique. The most useful application of this procedure may be the detection of endothelial loss in small arteries or other vascular beds that are not readily accessible for examination by scanning electron microscopy or other en face morphological techniques. For example, preliminary data indicate that this approach is useful in detecting the loss of endothelial cells in the mesenteric arteries of acutely hypertensive rats. These results suggest that sparse endothelial denudation does indeed occur in this setting, an exhaustion study would be required to obtain a comparable quantitative assessment by morphology.

The conclusion that this is a quantitative measure of denudation needs to be tempered by the evidence that detectable $^{111}$In label from platelets can also be taken up in an accelerated fashion by the intact endothelium covering apparently uninjured regions of the vessel. The increase in activity over intact endothelium of animals subjected to vascular injury could not be accounted for by the nonspecific attachment of $^{111}$In not bound to platelets, but was inhibited by pretreatment of platelets with aspirin. Thus the activity appears to be derived from labeled platelet components themselves. In contrast to reports of human platelets, rat platelets treated with collagen or calcium ionophore release considerable $^{111}$In activity (Table 1). Moreover, while in human platelets the bulk of the indium localizes in the cytoplasm rather than the granules, this value appears to vary considerably in other species. For example, rabbit platelets have a significant percentage of releasable platelet $^{111}$In. Thus, the appearance of radioactivity in regions of the vessel with intact endothelium suggests that released platelet products have somehow become associated with the wall.

At least three explanations can be offered to explain uptake of material released from the labeled platelets. First, the labeling of intact endothelium could represent uptake of materials released from platelets interacting either with the catheter or the denuded surface (Figure 5). At first, this seems unlikely since we could not reproduce the labeling by infusion of $^{111}$In-labeled material released from labeled platelets. On the other hand, the local concentrations of released material in the outer layers of flow in vivo could be much higher than that achieved in our experiment where preformed releasate was injected. This released material might also bind with plasma proteins in vitro before injection and consequently not bind in vivo. Moreover, pretreatment of platelets with aspirin did not prevent adherence to the denuded surface but did abolish uptake of label by the intact surface. This is most readily explained by the capacity of ASA to inhibit platelet release, but not adherence. Second, the labeling could represent some
form of injury to the platelets resulting in transient interactions of platelets with the intact surface. Third, the labeling could represent some morphologically nonapparent injury to the endothelium, resulting in transient interactions with the platelets. Since we did not see platelets on the intact surface, both the second or the third hypothesis would require that a transient interaction occur between these two cell types, inducing the release of platelet contents and the subsequent uptake by the endothelium. Such a transient interaction involving release of platelet granule has not been described in vivo. Again, the effect of aspirin could be explained by inhibition of release.

Thus interactions between platelet constituents and the vessel wall could occur by mechanisms other than overt deposition of intact platelets, and in light of the above results, it seems likely that a portion of platelet products released proximally may be present on or taken up by apparently intact endothelial surfaces and may not necessarily reflect defective endothelial integrity. In terms of spontaneous in vivo injury, it is interesting to speculate as to whether local flow disturbances such as may be found around a raised intimal lesion or at a site of stenosis could also lead to released platelet proteins that would presumably be in relatively high concentrations immediately downstream from this site.

References


Index Terms: endothelial denudation • indium-111 • platelets • aorta • aspirin

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Arterioscler Thromb Vasc Biol. 1986;6:305-311
doi: 10.1161/01.ATV.6.3.305

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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